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DETERMINATION OF GENETIC RELATEDNESS IN KELAMPAYAN PLANTLETS REGENERATED FROM TISSUE CULTURE USING DAMD MARKERS

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DECLARATION

I hereby declare that this is based on original work except for quotations and citations which have been acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UNIMAS or other institutions.

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LIST OF ABBREVIATIONS

bp	basepair
cm	centimeter
CTAB	Cetyl Trimethyl Ammonium Bromide
DNA	deoxyribonucleic acid
dNTPs	deoxynucleotide-triphosphates
kb	kilo basepair
m	meter
$MgCl_2$	Magnesium chloride
min	minutes
ml	milliliter
mm	millimeter
mM	milimolar
µl	microliter
PCR	Polymerase Chain Reaction
pH	potential of Hydrogen
pmol/µl	picomolar per microliter
POPGENE	Population genetics
TAE	Tris- Acetate –EDTA
U	Unit
V	Volt
%	percent
^oC	Celcius

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Determination of Genetic Relatedness in Kelampayan Plantlets Regenerated from Tissue Culture using DAMD Markers

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ABSTRACT

Neolamarckia cadamba and *Neolamarckia macrophylla* belongs to Rubiaceae family and locally known as white and red kelampayan respectively. They are economically important woody trees. These trees are large, deciduous and fast growing tree species. Most important of any *in vitro* propagation system is mass multiplication of plantlets that are genetically homogenous and phenotypically uniform. Modified CTAB method was used to extract the DNA. Directed amplification of Minisatellite-region DNA primers namely M13, YNZ, and 33.6 primers was used as a tool to assess the genetic relatedness of *in vitro* propagated *N. cadamba* and *N. macrophylla* hardened explants. The presence or absence of the amplification product showed allelic variation among individuals after visualizing as a band during electrophoresis. Cluster analysis of DAMD-PCR data using UPGMA (unweighted pair group method with arithmetic average) revealed the genetic relatedness among the species. A total of 21 bands and 24 bands were produced respectively. Mean gene diversity of *N.cadamba* and *N.macrophylla* is 0.1825 and 0.0926 respectively. Shannon index is 0.2632 and 0.1324 respectively. The low figure of both mean of gene diversity and Shannon index obtained shows that *N. cadamba* are related to each other while *N. macrophylla* samples are closely related. The UPGMA cluster analyses revealed *N. macrophylla* samples are highly related among the samples but two samples; MGR10-6 and MGR2-2 belongs to different clones shows close relatedness. The close relatedness among the samples indicates that tissue culture generated samples of both the kelampayan species shows very little polymorphism and therefore *in-vitro* regenerated plantlets in this study maintain their genetic integrity.

Keywords: Kelampayan, Random Amplified Polymerase DNA, *in vitro* propagation, genetic relatedness.

ABSTRAK

Neolamarckia cadamba dan Neolamarckia macrophylla tergolong dalam keluarga Rubiaceae dan nama tempatan masing-masing dikenali sebagai kelampayan putih dan merah. Pokok-pokok ini amat penting dari segi ekonomi. Hal ini kerana pokok ini besar, berdaun lebat serta tumbesaran yang cepat. Perkara penting dalam sistem pembiakan vitro adalah anak pokok yang seragam secara genomik dan fenotip meningkat dengan banyak. Kaedah CTAB telah digunakan untuk mengekstrak DNA. Primer M13, YNZ, dan 33.6 telah digunakan untuk menilai pertalian genetik diantara N. cadamba dan N. macrophylla. Analisis kelompok data DAMD menggunakan UPGMA telah menunjukkan pertalian genetik antara spesies. Min persamaan genetic bagi kumpulan pokok N.cadamba dan N. macrophylla ialah 0,1825 dan 0,0926. Manakala indeks Shannon adalah 0.2632 dan 0.1324. Analisis kelompok UPGMA di antara anak pokok tersebut menunjukkan bahawa kesemua anak pokok kedua- dua spesis tersebut menunjukan hubungan rapat dari segi genomic. Namun dua sampel dari N. macrophylla (MGR10-6 dan MGR2-2) menunjukan hubungan rapat walaupun diperolehi dari klon yang berbeza. Pertalian erat di kalangan sampel menunjukkan kedua-dua spesies kelampayan menunjukkan 'polymorphism' yang rendah dan oleh itu, tanaman kaedah in vitro yang digunakan dalam kajian ini mengekalkan integriti genetik mereka.

Kata kunci:Kelampayan, Hubungan genetic, Random Amplified Polymerase DNA, Kaedah in vitro.

CHAPTER I

INTRODUCTION

This research focus on the determination of the genetic relatedness of kelampayan plantlets: *Neolamarckia cadamba* and *Neolamarckia macrophylla*. Both this species belongs to Rubiaceae family, is an economically important species. It is commonly known as kelampayan, important for pulp and paper industry.

It is widely distributed from India, Nepal, through Thailand and Indo-China and eastward in the Malaysian Archipelago to Papua New Guinea (Joker, 2000). The tree is mostly found below 1000 m altitude and places where more than 1500 mm rain/ year are noted and also it can grow in dry areas with as little as 200 mm rain/year within the area of its natural distribution (Joker, 2000).

There are several studies used to assess the genetic relatedness of *in vitro* derived clones namely by karyological analysis, isozyme markers and polymerase chain reaction in conjunction with short primers. But most strategies have its limitations. For example, karyological analysis cannot reveal alterations in specific genes or small chromosomal rearrangements (Isabel *et al.*, 1993). Though isozyme markers provide a convenient method for detecting genetic changes, but are subjected to ontogenic variations. Also, only DNA regions coding for soluble proteins can be sampled.

PCR with short primers of arbitrary sequence (Williams *et al.,* 1990) shown to be sensitive for detecting variations among individuals between and within species (Carlson *et al.,*

1991; Roy *et al.,* 1992). This technique requires no prior knowledge of the genome and it needs only a small amount of DNA. Using this technique polymorphism can be detected in closely related organism.

Micropropagation of tree species offers rapid means of producing clonal planting stock for afforestation, woody biomass, and conservation of elite germplasm. Also, it is an alternative tool for crop improvement (Vasil, 1988). Therefore, tissue culture plays important role in replanting forest trees such as *N. cadamba* and *N. macrophylla* (Ndoye *et al.,* 2003).

Plantlets derived from *in vitro* culture might exhibit somaclonal variation (Larkin and Scowcroft, 1981) which is often heritable (Breiman *et al.,* 1987). The variations might be due to genotypes used (Breiman *et al.,* 1987), pathways of regeneration, and parameters used during *in vitro* culture such as morphology and cytology (Swedlund and Vasil, 1985) and molecular studies (Breiman *et al.,* 1989; Chawdhury *et al.,* 1994; Shenoy and Vasil, 1992).

Kelampayan improvement through traditional method is a slow, time and labor consuming process. So the optimization regeneration system is an important process and is considered as a prerequisite step towards plant transformation. Cell and tissue culture related to variability and selection efficiency are two essential components of the molecular breeding program (Lichtenstein and Draper, 1985).

The genetic analysis of these species derived from tissue culture is important because it will enable to choose the best quality trees with superior growth potential. Higher multiplication rates of selected tree genotypes are possible with micropropagation with uniform genotypes, resulting in short-term production gains. *N. cadamba* has been micropropagated because of its importance in the pulp and mining industries. Knowledge on the genetic stability of the proposed

samples will enable to plant more trees that show high good quality trees. Good quality includes larger width, high and faster growth.

However, the pressure for its valuable timber and fodder due to human activities has increased lately. Many tree felled off due to its importance in pulp and paper industry. Therefore, importance of replanting this tree has increased lately. Total of 18,851 ha of kelampayan trees planted in Sarawak which are approximately 8 % of overall species planted (Forest Department of Sarawak, 2012).

Today demand for plants with superior growth potential is high. Thus application of clonal propagation is widely used to produce high multiplication rates of uniform genotypes. Therefore, the objective of this study is to assess the genetic relatedness of *N. cadamba* and *N. macrophylla* plantlets derived from tissue culture with DAMD markers and its ability to form a uniform genetic profile. Also, to provide knowledge on genetic variance among the tested samples.

CHAPTER II

LITERATURE REVIEW

2.1 *Neolamarckia cadamba*

Neolamarckia cadamba or locally known as kelampayan is a timber species belongs to Rubiaceae family. The tree can grow up to 45 m tall and with diameter of 100 – 160 cm (Joker, 2000). The crown is open and round and bears drooping branches. The stem is straight and more or less cylindrical. Kelampayan has its flowers in orange color, small in dense and with globose heads while its leaves are up to 32 cm long (Joker, 2000).

N. cadamba (Roxb.) Bosser is a multipurpose tree known for many uses includes paper, wooden shoes, match sticks, plywood, pencil slats, wood carving, packing cases and boxes, and also splint manufacture (Lugo and Figueroa, 1984). The flowers of *N. cadamba* are offered at hindu shrines, and the fruits are eaten in India (Lugo and Figueroa, 1984).

Figure 2.1: Flowers of *N. cadamba*

Figure 2.2: Green leaves of *N. cadamba*

This tree is a fast growing tree and suitable for reforestation conservation especially in watersheds and also for windbreaks in agroforestry systems (Joker, 2000). *N. cadamba* is known for its lightweight hardwood which is poor durability therefore mainly used for pulp (producing low and medium quality paper) and veneer plywood. In addition, the bark and leaves have various medicinal uses such as astringent anti-hepatotoxic, antidiuretic, wound healing, antiseptic and anthelmintic (Patel and Kumar, 2008).

2.2 *Neolamarckia macrophylla*

Neolamarckia macrophylla (Roxb.) Bosser is a member of Rubiaceae family or more commonly known as red kelampayan. It has reddish green hairy leave surface and its stem is in dark red. Red kelampayan is one type of fast-growing plants with a stem circumference more than 150 cm (diameter greater than 50 cm) (Halawane *et al.*, 2012). *N. macrophylla* is a tree with straight trunk that is free from branch up to 70% -80%. It drops the bottom leaves to grow straight up without branching and has smooth texture and straight wood grain. This tree grows well in locations with a height of 10-1000 m above sea level. Ability to grow on degraded land is also quite good, and even can be used as a buffer zone for the conservation purpose due to its deep roots (Halawane *et al.*, 2012).

With the red color and a smooth texture, this wood can be used as a raw material for plywood, furniture, plywood (veneer), home accessories and more. Also used as the raw material of short fiber pulp with medium quality.

Figure 2.3: Seven-month old *N. macrophylla* leaf

(Source: http://jabon.web.id/jawaban-atas-pertanyaan-dari-jabon-merah/)

Table 2.1: Morphological characteristics differences of *N. macrophylla* and *N. cadamba*

Characteristics	***N. macrophylla***	***N. cadamba***
Young leaf buds	Red	Brown
Basal leaves	Sharp-pointed	Flat
Primary leaf veins	Red	Yellowish green
Young stems	Blackish red	Brownish green
Mature tree trunks	Black	Brown grey
Fruit color	Ripen fruit is reddish brown	Ripen fruit is yellow

Source: Halawane *et al.*, 2012.

2.3 Directed Amplification of Minisatellite-region DNA (DAMD) Marker

Among the PCR based DNA marker systems, RAPD, DAMD and ISSR are commonly and extensively used tools for assessment of variability in crops. Due to their ease, rapidity and reliability, this marker system is efficient for analysis of molecular differentiation and for resolving taxonomic problems in plants (Ranade et al., 2006). Repetitive DNA sequences such as microsatellites, minisatellites, and satellites can be of great value in assessing a high level of polymorphism as they distributed throughout the genomes analyzed thus far.

Directed amplification of minisatellite-region DNA (DAMD) uses VNTR core sequences such as M13 as primers in PCR reactions (Heath et al. 1993). Minisatellites are tandemly repeated DNA regions of eukaryotic genomes. It shows high levels of variation due to differences in the number of repeated units (Jeffreys *et al*. 1985). It involved an inversion of a portion of a minisatellite DNA array with a single primer makes PCR is possible for the amplification of minisatellite core region (Heath *et al*. 1993). Since minisatellite core sequences which are used as primers are longer than RAPD-PCR primers, DAMD-PCR can be effectively carried out at relatively high stringency reactions (Bebeli *et al*. 1997).

However, this marker does have its limitation in terms of uncertain homology of co-migrating fragments in gel electrophoresis and reproducibility. This can be minimized by carefully adjusting the reaction and detection conditions. Table 2.2 shows the primers used in this study.

Table 2.2: List of primers used in this study

Primer code	Primer Sequence 5' to 3'	Nucleotide length
M13	5'- TTATGAAACGACGGCCAGT-3	19
33.6	5'- AGGGCTGGAGG - 3'	11
YNZ -22	5'- CTCTGGGTGTGGTGC- 3'	15

2.4 Tissue culture induced variations

Plant tissue culture has become one of the fundamental tools of plant science research. It is extensively employed in the production, conservation and improvement of plant resources. Therefore, *in vitro* culture techniques provide an alternative means of plant propagation and a tool for crop improvement (Vasil, 1988).

Tissue culture and *in vitro* plantlet regeneration systems provide an alternative production system for mass propagation of the desired plant genotype for commercial planting. These systems are applied when the selected genotype is required at mass scale, for commercial cultivation when the natural propagation does not meet the required demands.

However, *in vitro* techniques do induce somaclonal variations. According to Rathore *et al.,* (2011), the frequency of somaclonal variations varies with the source of explants and their regeneration pattern, media composition, and cultural conditions. These variations remain one of the major problems of many tissue-cultured plants (Rathore *et al.,* 2011). Uncontrolled and random spontaneous variations during culture process are major causes.

Nevertheless, variations are not always bad, it does sought for crop improvement by creation of novel variants through applications of advanced biotechnological tools (Rathore *et*

al., 2011). In addition, it induces variations in genetic manipulation of crops with phylogenic traits. Since the somaclonal variations in tissue culture plantlets are possible, therefore the need to test and verify the genetic stability of regenerated plantlets is important. The variations can be determined at the morphological, cytological, biochemical, and molecular levels. At present, molecular markers are used in the analysis of genetic fidelity of in vitro propagated plantlets.

The most important part of any *in vitro* propagation system is mass multiplication of plantlets that are genetically homogenous and phenotypically uniform. Several approaches have been applied for identifying variants among micropropagated plants. Molecular markers have been shown to enhance breeding efforts in annual and perennial crops, since they are not altered by major environmental factors.

CHAPTER III

MATERIALS AND METHODS

3.1 Plant Samples and Isolated DNA Samples

Hardened tissue cultured kelampayan leaves of *N. cadamba* and *N. macrophylla* were obtained from the MGene Propagation Sdn Bhd. There are total of six samples of *N. cadamba* and nine samples of *N. macrophylla.* Table 3.1 shows the list of samples.

Table 3.1: List of samples of *N. cadamba* and *N. macrophylla*

Species								
Neolamarckia macrophylla			***Neolamarckia cadamba***					
Clone 1	Clone 2	Clone 3	Clone 1	Clone 2	Clone 3	Clone 4	Clone 5	Clone 6
MGR 2-1	MGR 6-3A	MGR 10-1	MGK 03	MGK 04	MGK 05	MGK 06	MGK 08	MGK 10
MGR 2-2	MGR 6-3B	MGR 10-2						
MGR 2-6	MGR 6-4	MGR 10-6						

Total genomic DNA of *N. cadamba* and *N. macrophylla* was extracted by following modified CTAB method by Doyle and Doyle (1990). Purification of DNA sample is done by following protocol of Wizard® Genomic DNA Purification Kit (Promega). The presence of isolated genomic DNA from the leaf was determined by running a 0.8% agarose gel. The yield of DNA was measured using Nanodrop 2000 Spectrophotometer (Thermo Fisher Scientific, USA). The purity of DNA was determined by calculating the ratio of absorbance at 260 nm to that of 280 nm.

3.2 PCR Amplification

3.2.1 PCR Condition

Prior to begin the PCR reaction, a total of 25 µl PCR mixtures were prepared. PCR amplifications were performed on a Gradient Palm-Cycler^{TM} (Corbett Research, Australia). The PCR products were checked using 1.5% agarose gel and run simultaneously with 100 bp or 1 kb DNA ladder (Promega, USA). The PCR optimization was carried out to determine the optimal condition for this analysis. The PCR reaction mixture profile in a total volume of 25 ul is shown in Table 3.1.

Table 3.2: PCR ingredients for 25 µl reaction mixture

Reagent	**Concentration**
Double distilled Water	-
PCR buffer	10 ×
dNTPs	2 mM
$MgCl_2$	50 mM
Primer	2.5 pmol
Taq DNA polymerase	0.5 U
Template DNA	15 ng/ µl

The thermal cycling profile used for all three primers M13, 33.6 and YNZ-22 for preliminary PCR optimization step is shown in Table 3.2. The PCR reaction conditions used to generate *N. cadamba* and *N. macrophylla* DNA profiles using M13 universal primer is shown in Table 3.3.

Table 3.3: A thermal cycling profile used for preliminary PCR optimization step

Parameter	Temperature (^{o}C)	Timing (min)	
Initial Denaturation	94.0	2	
Denaturation	94.0	1	35 cycles
Annealing	48.0	1	
Extension	72.0	2	
Final Extension	72.0	10	
Hold	4.0	∞	

Table 3.4: PCR reaction condition with M13 minisatellite universal primer.

Reagent	Concentration	Volume (µl)
PCR buffer	1×	2.5
dNTPs	2 mM	2.5
$MgCl_2$	50 mM	1.5
Primer	2.5 pmol	6.0
Taq DNA polymerase	0.5 U	4.0
Template DNA	15 ng/ µl	1.0
ddH_2O		7.5
Total volume		**25.0**